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X-Chromosome Inactivation Is Mostly Random in Placental Tissues of Female Monozygotic Twins and Triplets

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Patterns of X-chromosome inactivation in chorion, amnion, and cord from 79 pairs of twins were examined. Seven sets of triplets were included in the analysis, both as twin pairs and triplets. Twins were stratified as dizygotic (DZ), monozygotic (MZ), monochorionic, and dichorionic and were selected for birth weight discordance, discordance for congenital anomalies, twin-twin transfusion syndrome, and various patterns of vascular anastomosis.

X-inactivation was predominantly symmetric. Chorion was the most likely tissue to show asymmetric X-inactivation and was found most frequently in MZ dichorionic twins. There was no correlation of X-inactivation pattern with the selected clinical criteria. This study does not confirm that asymmetric X-inactivation in embryonic tissues is a common phenomenon in female twins, including monozygotic twins.

KEY WORDS: twins, triplets, X-chromosome inactivation, monozygotic, dichorionic, monochorionic

INTRODUCTION

Several monozygotic (MZ) female twin pairs have been reported who are discordant for the phenotypic expression of X-linked diseases (Table I). There are at least two patterns of asymmetric X-inactivation in these twin pairs. In one pattern, both twins show asymmetric X-inactivation, but in opposite or reciprocal directions; the twin with the predominantly inactivated paternal X-chromosome manifests the disease. In the other pattern, the affected twin has predominantly inactivated the paternal X-chromosome, while the unaffected twin shows symmetric X-inactivation. There do not appear to be cases in which both members of an MZ female pair show symmetric X-inactivation, nor pairs in which asymmetric X-inactivation has taken place in the same direction in both twins. At least in MZ female twins with phenotypic X-linked disease expression, one twin is affected and the other is clinically normal, however, see also Goodship et al. [1996]. This phenomenon of discordant X-inactivation within twin pairs has led to the concept that discordant X-inactivation might actually trigger the MZ twinning process in the case of symmetric X-inactivation, or be an indication of unequal allocation of blastomeres in the cases of twin pairs with asymmetric/symmetric X-inactivation.

This hypothesis has been tested using X-linked methylated DNA markers on umbilical cord tissue and blood samples from normal female MZ pairs [Goodship et al., 1992; Richards et al., 1990a,b]. However, it is recognized that results on peripheral blood leucocyte DNA may not be representative of X-inactivation in somatic cells; for monochorionic MZ twins, patterns of X-inactivation in blood leucocytes may reflect presence or absence of transfused stem cells via inter-fetal vascular anastomoses.

To study patterns of X-inactivation in female MZ twins, we used DNA derived from chorion, amnion and umbilical cord. This project was possible because all like-sex newborn twins in our institution are tested for zygosity at birth.

MATERIALS AND METHODS

All placentas of spontaneous and induced like-sex twin and higher-order multiple pregnancies delivered at University of Alberta Hospitals are sampled for DNA analysis of zygosity. From the female twin pairs and triplet sets, we chose a variety of zygosities and clinical settings in which to document patterns of X-inactivation (Table II).

DNA was extracted from fresh samples of chorionic parenchyma, amnion, and umbilical cord, using standard methods [Old, 1986]. Zygosity was determined by molecular testing with VNTR probes 3'HVR [Higgs et al., 1986], YNH/24 [Nakamura et al., 1987], and

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TABLE I. Asymmetric X-inactivation in Female Monozygotic Twin Pairs With Clinical Expression of X-Linked Diseases*

Author	Disease	Inactivation detection method	Affected twin DNA source		Normal twin DNA source		Possible mechanism	Pedigree
			F	L	F	L		
Tuckerman et al. [1985]	XLMR	BRdU incorporation	—	Sk to wild	—	Sk to mutant	RSk, pre-T	
Burn et al. [1986]	DMD	Somatic cell hybrids	Sk to wild	—	Sk to mutant	—	RSk, pre-T	? de novo, gonadal mosaic
Richards et al. [1990]	DMD	DXS255	Sk to wild	Sk to wild	Sk to mutant	Sk to mutant	RSk, pre-T	maternal carrier
Lupski et al. [1991]	DMD	DXS255	—	Sk to wild	—	R	UBA, post-T	? de novo, normal twin has affected boy
Abbadi et al. [1992]	DMD	DXS255	—	Sk to wild	—	Sk to mutant	RSk, pre-T	Mother, sister carriers; affected brother
Jorgensen et al. [1992]	RGCB	DXS255	Sk to wild	R	Sk to mutant	R	UBA post-T, blood chimerism	?
Winchester et al. [1992]	Hunter disease	DXS255 (M27β)	Sk to wild	Sk to wild	—	R	UBA, post-T	Maternal carrier
Kruyer et al. [1993]	XLMR	DXS255 (M27β)	—	Sk to wild	—	Sk to mutant	RSk, pre-T	
Levade et al. [1991]	Fabry	Hair root α-galactosidase A	—	—	—	—	?	Son of unaffected twin hemizygous

*F, fibroblasts; L, leucocytes; XLMR, X-linked mental retardation; Sk, skewed; RSk, reciprocal skewed X-inactivation; pre-T, occurs before twinning; DMD, Duchenne muscular dystrophy; R, random; UBA, unequal blastomere allocation; post-T, occurs after twinning; RGCB, red-green color blindness. Placental chorionicity was not known in any of these cases.

INS/310 [Bell et al., 1981]. X-inactivation studies were carried out using methylation analysis at the DXS255 locus using the M27β probe [Fraser et al., 1989]. DNA was initially digested with PstI according to the manufacturer's instructions. Aliquots of this sample were then subjected to a second digestion with either MspI or HpaII [Abrahamson et al., 1990] to detect differences in methylation. Assessment of relative band intensity on autoradiography following Southern blotting was used to classify the X-inactivation pattern in the twin pairs as symmetric, slightly asymmetric, and asymmetric for each tissue (Figs. 1, 2). For the data analysis both symmetric and slightly asymmetric X-inactivation were considered symmetric X-inactivation. Triplet sets were analyzed both as a separate group and as sets of three twin pairs.

RESULTS

Table II summarizes each of the cases. The female twin pairs and triplet sets were stratified as dizygotic (DZ), monozygotic (MZ), and monochorionic and dichorionic, with varying degrees of birth weight discordance, various patterns of vascular anastomoses in monochorionic pairs and sets, for presence of twin-twin transfusion, and for discordance for major congenital anomaly. No consistent pattern of X-inactivation was found. In two of the pairs discordant for major malformation, the X-linked probe was non-informative as both twins were homozygous at the DXS255 locus.

Symmetric/symmetric X-inactivation was the most common observed pattern. Both patterns of asymmetric X-inactivation were seen, but there was little stratification of patterns according to the clinical status of the twins and triplets. In addition to reciprocal asymmetric X-inactivation, we also noted asymmetric X-inactivation in the same direction in chorionic DNA of two twin pairs.

Tables III–IX analyze the patterns of X-inactivation in the three tissues and in different settings of zygosity, chorionicity, and clinical outcomes.

Table III shows that there is more asymmetric X-inactivation in MZ dichorionic than MZ monochorionic twin pairs, but this does not reach statistical significance. For all tissues examined, symmetric X-inactivation predominated (Table IV); asymmetric X-inactivation was found most frequently in the chorion of MZ dichorionic twins (30%) and least frequently in the cords of MZ monochorionic twins (7%). For all pairs tested, one-half of MZ dichorionic twins had one twin with asymmetric X-inactivation (Table V), whereas only 10% of MZ monochorionic pairs had one twin with asymmetric X-inactivation.

Table VI confirms the high frequency of pairs of MZ dichorionic twins with asymmetric X-inactivation found in chorion and umbilical cord DNA. Low rates of asymmetric X-inactivation were found in all tissues of MZ monochorionic twin pairs.

Table VII analyzes patterns of X-inactivation in MZ twin pairs by birth weight discordance. In the MZ dichorionic twin pairs, X-inactivation was most frequently random in the smaller twin, and a high rate of asymmetric X-inactivation was only found in the chorionic DNA of the larger twins. In MZ monochorionic

TABLE II. Patterns of X-inactivation in Twin Pairs and Triplet Sets (Analysis by Placental Status and Clinical Outcomes)*

Case No.	Chorion	Amnion	Cord	GD%[a]	Anast.	Venous share	Clinical status
DZ							
1	S/S	S/S	—	4			
2	A/S	—	—	7			
3	S/S	S/S	S/S	14			
4	S/S	S/S	—	18			
5	S/S	S/S	S/S	24			
MZ, DC							
6	S/S Reciprocal	S/S	S/S Reciprocal	3			
7	A/S	S/S	—	6			
8	Homozygous			6			B had left heart hypoplasia
9	A/S	S/S	S/S	16			
10	S/S	S/S	S/A	20			
MC, DA							
11	A/S	S/S	S/S	0	None	Equal	
12	S/S	S/S	S/S	2	None	A > B	
13	A/A, same	A/S	A/S	14	a→a	A > B	B had omphalocele
14	S/S	S/S	S/S	15	a→a v→v a→v	Equal	
15	A/A, same	S/A	—	17	None	Equal	
16	S/S	S/S	S/S	18	None	Equal	
17	S/S	A/A, Reciprocal	S/A	18	a→a a→v	A > B	
18	S/S	S/S	S/S	32	None	A > B	
19	S/A	A/S	S/S Reciprocal	34	a→a a→v	A > B	
20	S/S, Opposite	—	S/S	34	None	A > B	
21	S/S	S/S	S/S	46	a→v	A > B	Double fetal death, TTT
22	Homozygous			—	a→v	A > B	TTT; B had anencephaly
23	S/S	—	—	53	a→v	A > B	Double fetal death, TTT
MC, MA							
24	S/S	—	—	8	None	Equal	
MZ triplet sets							
25	A/S/S	—	S/S/S	—	a→v	Unequal	A, B, double fetal death, TTT. C was DC to A and B, dying of prematurity
26	S/S/A	S/S/A	S/S/A	—	a→v	Unequal	B, C, double fetal death, TTT. A was DC to B and C, dying of prematurity
27	S/S/S	S/S/S	S/S/S	—	a→v	Unequal	MC, TA, B and C dying of TTT, A dying of prematurity

*DZ, dizygotic; MZ, monozygotic; DC, dichorionic; MC, monochorionic; DA, diamniotic; MA, monoamniotic; TA, triamniotic; S, symmetric; A, asymmetric; a→a, arterio-arterial; v→v, veno-venous; a→v, arterio-venous; TTT, twin-twin transfusion.
[a]Large-small/Large × 100. By convention, the larger twin is twin A in all pairs.

twin pairs, the same ratios of symmetric and asymmetric X-inactivation were seen in the larger and smaller twin; asymmetric X-inactivation was least frequent in the cords of large and small MZ monochorionic twins.

In MZ monochorionic twin pairs and triplet sets, no significant differences between X-inactivation patterns were found in donors and recipients in prenatal twin-twin transfusion (Table VIII). In monochorionic, MZ pairs analyzed by severity of growth discordance (because of twin-twin transfusion or unequal venous sharing of the placenta), symmetric X-inactivation was most frequent in the pairs with severe growth discordance (Table IX).

In the triplet sets containing dichorionic and monochorionic combinations, there were no differences in the patterns of X-inactivation between the pairs of twins within triplets of the one zygote in which two consecutive twinning events have occurred at different times.

DISCUSSION

MZ twin females discordant for X-linked diseases are well recognized. X-inactivation studies carried out on this special group of discordant female MZ twins have shown two patterns of X-inactivation; there was reciprocal asymmetric X-inactivation in two sets of MZ

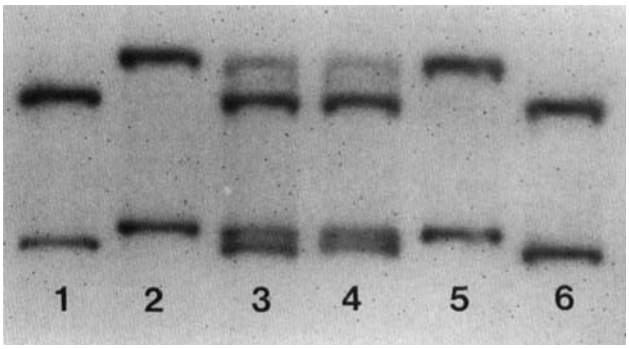

Fig. 1. Case No. 9. Methylation analysis showing S/S X-inactivation in amnion in a twin pair. DNA from twin A **(lanes 1–3)** and twin B **(lanes 4–6)** was digested with Pst I alone (lanes 1 and 6), Pst I and Msp I (lanes 2 and 5), and Pst I and Hpa II (lanes 3 and 4) and probed with M27β.

TABLE III. Analysis of X-inactivation Status of Each Embryo, Incorporating the Triplets as Pairs of Monochorionic and Dichorionic Monozygotic Twins (All Tissues, No. [%])

	X-inactivation pattern		
Group	S	A	Total
Twins			
DZ	21 (95)	1 (5)	22 (100)
MZ, DC	21 (78)	6 (22)	27 (100)
MZ, MC	72 (85)	13 (15)	85 (100)
All MZ twins	93 (83)	19 (17)	112 (100)
All twins	114 (85)	20 (15)	134 (100)
Triplets			
MZ, DC	2	3	5
MZ, MC	18	1	19
All triplets	20	4	24

twins discordant for Duchenne muscular dystrophy (DMD) and red-green color blindness, respectively [Richards et al., 1990a,b; Jorgensen et al., 1992; Zneimer et al., 1993], and asymmetric/symmetric X-inactivation in two sets discordant for Duchenne muscular dystrophy and Hunter syndrome, respectively [Lupski et al., 1991; Winchester et al., 1992]. Twins who both show asymmetric X-inactivation and express the gene for the X-linked disease have not been reported. It is postulated that reciprocal asymmetric X-inactivation occurs when the twinning process splits the inner cell mass equally soon after X-inactivation when there are only a few cells in the inner cell mass [Nance, 1990]. The second scenario of symmetric/asymmetric X-inactivation might occur when the twinning process occurs later and a relatively small proportion of the inner cell mass is extruded through a break in the zona pellucida after X-inactivation has occurred. These cells might be expected by chance to show an asymmetric X-inactivation pattern, as they represent a small sample size from a clonal area of the parent inner cell mass. The new inner cell mass would then show catch-up growth giving rise to a second embryo with apparent asymmetric X-inactivation [Lupski et al., 1991] who might be of lower birth weight than the co-twin and show increased size of cell clones. This would have little effect on the random X-inactivation of most of the remaining inner cell mass that formed the non-diseased twin. In extensive studies of X-inactivation in the mouse, Tan et al. [1993] showed that X-inactivation occurs at different times in gestation in different tissues. They did not study X-inactivation in amnion and umbilical cord.

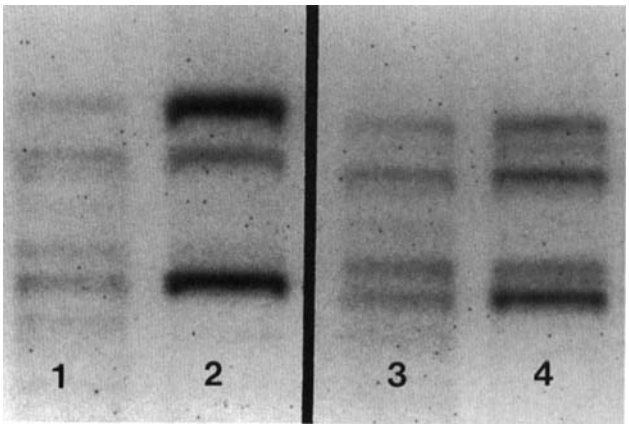

Fig. 2. Case No. 7. Methylation analysis showing S/A X-inactivation in chorion and S/S X-inactivation in amnion of a twin pair. DNA isolated from chorion (**lane 1,** twin A; **lane 2,** twin B) and amnion (**lane 3,** twin A; **lane 4,** twin B) digested with Pst I and Msp I (lanes 1 and 4), Pst I and Hpa II (lanes 2 and 3), and probed with M27β.

This project set out to investigate X-inactivation patterns in different embryonic tissues from DZ and MZ female twins who did not have X-linked disease, but who showed varying degrees of discordance for birth weight, congenital anomalies, and the presence of twin-twin transfusion. Discordance for weight was selected to investigate whether a twin developing from a small inner cell mass [Lupski et al., 1991] might show reduced birth weight and asymmetric X-inactivation. Studies on X-inactivation from umbilical cord of MZ twins [Goodship et al., 1992] have demonstrated that there may be differences in X-inactivation activation patterns between MZ and DZ twin pairs. However, even DZ twins showed some differences in X-inactivation patterns. Because different tissues might show different patterns of X-inactivation in normal individuals [Brown and Brown, 1993], we elected to look at three different tissues.

No consistent pattern of X-inactivation was found in the twin pairs and triplet sets. Symmetric/symmetric X-inactivation was the most common pattern seen. Chorion was the most likely tissue to show any asymmetric X-inactivation. When the samples were analyzed for each embryo, the X-inactivation pattern for chorion was similar to that found in cultured fibroblasts [Brown and Brown, 1993]. They found random X-inactivation [50:50, 40:60, 30:70) in 58% of cultures, slight asymmetric X-inactivation (20:80) in 14% of cultures and asymmetric X-inactivation (10:90, 0:100) in 26%. The corresponding values in chorion in this study were 65% symmetric X-inactivation, 16% slight asymmetric X-inactivation, and 20% asymmetric X-inactivation. However, no member of a DZ twin pair showed asymmetric X-inactivation and only one member of a

TABLE IV. Analysis of X-inactivation Status in Each Embryo by Tissue, Incorporating the Triplets as Pairs of Monochorionic and Dichorionic Monozygotic Twins (No. [%])

	Tissue X-inactivation pattern							
	Chorion		Amnion		Cord		Total	
Group	S	A	S	A	S	A	S	A
Twins								
DZ	9 (90)	1 (10)	8	0	4	0	21 (95)	1 (5)
MZ, DC	7 (70)	3 (30)	8 (89)	1 (11)	6 (75)	2 (25)	21 (78)	6 (22)
MZ, MC	27 (82)	6 (18)	20 (80)	5 (20)	25 (93)	2 (7)	72 (85)	13 (15)
All MZ	34 (79)	9 (21)	28 (82)	6 (18)	31 (89)	4 (11)	93 (83)	19 (17)
All twins	43 (81)	10 (19)	36 (86)	6 (14)	35 (90)	4 (10)	114 (85)	20 (15)
Triplets								
MZ, DC	1	3	0	2	2	2	3	7
MZ, MC	4	1	4	0	5	0	13	1
All triplets	5	4	4	2	7	2	16	8

DZ twin pair showed slight asymmetric X-inactivation in chorion. X-inactivation is random in chorionic mesoderm of singleton female fetuses [Harrison, 1989; Migeon et al., 1985], and our study largely confirmed this in MZ twin females. The X-inactivation patterns in cord and amnion were similar. All members of DZ twin pairs showed symmetric X-inactivation in cord and amnion.

Of the MZ twins, amnion and chorion showed 83% and 67% symmetric X-inactivation patterns, while 12% and 18%, respectively, showed asymmetric X-inactivation. The remainder showed slight asymmetric X-inactivation. The previously published series showed similar ratios for asymmetric X-inactivation in cord, but rather more samples with slightly asymmetric X-inactivation patterns than in the present series [Goodship et al., 1992]. Only one set of twins (MZ monochorionic) showed reciprocal asymmetric X-inactivation in amnion. In chorion and cord, the pattern was asymmetric/symmetric X-inactivation. However, asymmetric X-inactivation was more common in dichorionic than monochorionic MZ twins.

When the data were analyzed by MZ twin and triplet sets, there was again no definite X-inactivation pattern seen with MZ dichorionic and MZ monochorionic pairs or with twins discordant for birth weight in any of the tissues examined. We did not confirm the hypothesis that the smaller twin in an MZ twin pair might show an asymmetric X-inactivation pattern. We found an apparent difference in X-inactivation patterns between monochorionic MZ and dichorionic MZ twins, suggesting that timing of the twinning process (earlier in dichorionic twins) may influence the pattern of X-inactivation. Pairs with twin-twin transfusion did not show any prominent pattern of X-inactivation. These results are consistent with the theory of Lupski et al. [1991] that patterns of X-inactivation in MZ twins are more likely a consequence of unequal splitting of the inner cell mass during the twinning process rather than as a cause of twinning.

While it is well documented that MZ twins discordant for X-linked diseases may show differences in X-inactivation patterns, our data do not confirm that asymmetric X-inactivation in embryonic tissues is a common phenomenon in female MZ twins. Although there is an excess of asymmetric X-inactivation patterns in MZ twins compared to DZ twins, the commonest pattern is symmetric/symmetric X-inactivation. It is possible that the presence of an abnormal gene could influence the pattern of X-inactivation seen in discordant MZ twins by preferential inactivation of either the normal or mutant X-chromosome. This would then give rise to an inner cell mass containing an excess of cells expressing either the normal or mutant X-chromosome. Unequal splitting of the inner cell mass might then give rise to two new inner cell masses with differing X-inactivation patterns. It has been shown that, for some diseases, e.g., Wiscott-Aldrich syndrome, T and B cells in female carriers express the normal X-chromosome only. The mutant X-chromosome is inactive, giving rise to an apparent asymmetric X-inactivation pattern [Puck et al., 1990]. In contrast, cultured fibroblasts from female carriers of X-linked adrenoleucodystrophy express the abnormal X-chromosome, while the normal X-chromosome is inactive [Migeon et al., 1981]. However, in female carriers of Duchenne muscular dystrophy, myoblasts with either the mutant or normal X-chromosome active show equal ability to proliferate [Hurko et al., 1989]. Assuming that the same situation exists in the developing blastocyst, for this disease it would be expected that the inner cell mass would show symmetric X-inactivation.

Our data suggests that asymmetric X-inactivation in embryonic tissue in MZ female twins is uncommon,

TABLE V. Paired Patterns of X-inactivation, Incorporating Triplets, All Tissues (No. [%])

	X-inactivation, pairs		
Group	S/S	A/S and A/A	Total
Twins			
DZ	10 (91)	1 (9)	11 (100)
MZ, DC	11 (52)	10 (48)	21 (100)
MZ, MC	37 (79)	10 (21)	47 (100)
All MZ	48 (71)	20 (29)	68 (100)
All twins pairs	58 (73)	21 (27)	79 (100)
Triplets			
MZ, DC	3	7	10
MZ, MC	13	1	14
All triplets	16	8	24

TABLE VI. Patterns of X-inactivation in Twin Pairs, Analyzed by Tissues (Triplets Are Incorporated as Pairs, No. [%])

	X-inactivation, pairs, by tissues							
	Chorion		Amnion		Cord		Total	
Group	S/S	A/S A/A	S/S	A/S A/A	S/S	A/S A/A	S/S	A/S A/A
Twins								
DZ	4	1	4	—	2	—	10	1
MZ, DC	3 (37)	5 (63)	4 (67)	2 (33)	4 (57)	3 (43)	11 (52)	10 (48)
MZ, MC	14 (78)	4 (22)	10 (71)	4 (29)	13 (87)	2 (13)	37 (79)	10 (21)
All MZ	17 (65)	9 (35)	14 (70)	6 (30)	17 (72)	5 (28)	48 (71)	20 (29)
All twins	21 (68)	10 (32)	18 (75)	6 (25)	19 (79)	5 (21)	58 (73)	21 (27)
Triplets								
MZ, DC	1	3	0	2	2	2	3	7
MZ, MC	4	1	4	0	5	0	13	1
All triplets	5	4	4	2	7	2	16	8

TABLE VII. Patterns of X-inactivation in Monozygotic Pairs, Incorporating Triplets, Analyzed by Weight Discordance

	Chorion				Amnion				Cord				Total			
	Larger		Smaller		Larger		Smaller		Larger		Smaller		Larger		Smaller	
Group	S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A
Twins																
MZ, DC	3	5	8	0	4	2	6	0	5	2	6	1	12	9	20	1
MZ, MC	14	4	15	3	11	3	12	2	13	1	13	1	38	8	40	6
All MZ	17	9	23	3	15	5	18	2	18	3	19	2	50	17	60	7
Triplets																
MZ, DC	1	3	4	0	0	2	2	0	2	2	4	0	3	7	10	0
MZ, MC	4	1	5	0	4	0	4	0	5	0	5	0	13	1	14	0
All triplets	5	4	9	0	4	2	6	0	7	2	9	0	16	8	24	0

TABLE VIII. Monochorionic Twin-Twin Transfusion Pairs, Including Monochorionic Pairs From Monozygotic Triplets

Chorion				Amnion				Cord				All			
Donor		Recipient		Donor		Recipient		Donor		Recipient		Donor		Recipient	
S	A	S	A	S	A	S	A	S	A	S	A	S	A	S	A
Twins															
4	1	5	0	3	0	2	1	4	0	4	0	11	1	11	1
Triplets															
2	1	3	0	2	0	2	0	3	0	3	0	7	1	8	0

and, while it may play a role in the twinning process in a few female MZ twins, it does not play a significant role in the genesis of twinning in most MZ female twin pairs.

TABLE IX. Patterns of X-inactivation in Monochorionic Pairs, Analyzed by Severity of Growth Discordance (No. [%])

Growth discordance	S/S	S/A, A/A	Total
Twins			
<20%	12 (60)	8 (40)	20 (100)
>20%	12 (92)	1 (8)	13 (100)
Total	24 (73)	9 (27)	33 (100)
Triplets			
<20%	4	1	5
>20%	3	0	3
Total	7	1	8

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